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Serotonin activates angiogenic phosphorylation signaling in human endothelial cells

Ali Zamani^{b,*}, Zhican Qu^{a,b,c,*}^a Southern Research Institute, Birmingham, AL, USA^b Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, USA^c Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, USA

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ABSTRACT

Serotonin, a known neurotransmitter, also functions as an angiokine to promote angiogenesis. The majority of serotonin in the human body is stored in platelets, and platelet aggregation leads to significant release of serotonin in thrombotic tumor environment. We have investigated serotonin signaling in human endothelial cells. Through G-protein-coupled receptors, serotonin at physiologically relevant concentrations activated Src/PI3K/AKT/mTOR/p70S6K phosphorylation signaling, and this activation was similar to that seen with VEGF. This finding provides insight into the overlapping angiogenic signaling pathways stimulated by serotonin in tumor environment, and suggests one of the mechanisms underlying resistance to current VEGF-targeting antiangiogenic therapy against cancer.

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1. Introduction

The monoamine neurotransmitter serotonin (5-hydroxytryptamine (5-HT)) affects various neurological functions such as mood, sleep, and appetite [1]. In addition to its functions in the human central nervous system (CNS), 5-HT also plays important roles in peripheral tissues. It regulates primary homeostasis, gut motility, immune responses, and vasoconstriction. 5-HT also stimulates endothelial cells to release vasodilating substances, and acts as a “helper agonist” of platelet aggregation [2]. The biosynthesis of 5-HT is carried out primarily by serotonergic neurons and enterochromaffin cells in the gastrointestinal tract. Enterochromaffin cells produce and secrete approximately 90% of the total 5-HT in the human body. Under normal conditions, 5-HT cannot cross the blood–brain barrier [3]. Most peripheral 5-HT is incorporated into platelets and circulates in the bloodstream throughout the entire human vascular system [4].

Pro-thrombotic diseases such as cancer and *atherosclerosis* are associated with high platelet counts and abnormalities of platelet aggregation [5,6]. Endothelial cells are the main structural components of blood vessels, and are one of the first cell types that encounter 5-HT upon its release from activated platelets. The stim-

ulatory effects of 5-HT on the proliferation, migration, and tubulogenesis of endothelial cells [7–10] suggest that 5-HT plays a critical role in angiogenesis. In recent years, several studies have approved the concept that tumor growth is dependent upon angiogenesis [11,12]. Vascular endothelial growth factor (VEGF) has been considered as a major tumor angiogenic factor [13]. Several agents targeting VEGF or the VEGF receptor have been approved as antiangiogenic therapy for cancer. Unfortunately, the benefits of these drugs are limited and transient, and are often followed by resistance and the restoration of tumor progression [11,14]. These findings suggest that other angiogenic factors may also drive tumor angiogenesis. In the thrombotic environment of tumors, platelet aggregation frequently occurs and leads to significant release of serotonin, which may constitute one of the mechanisms of tumor angiogenesis.

2. Results

2.1. 5-HT activates signaling kinases in human endothelial cells

Several key signaling kinases have been shown to be involved in angiogenic signal transduction in human endothelial cells [15–18]. 5-HT has been demonstrated to have angiogenic potential. Therefore, we assessed if 5-HT could activate certain angiogenic signaling kinases. Seven signaling kinases were selected for the present study; Src kinase; phosphatidylinositol 3-kinase (PI3K); protein kinase B (AKT); mammalian target of rapamycin (mTOR); 70-kDa ribosomal protein S6 kinase (p70S6K); p44/42 mitogen-activated

* Corresponding authors. Addresses: Pharmacology and Toxicology Department, University of Alabama at Birmingham, Birmingham, AL 35209, USA (A. Zamani). Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, USA (Z. Qu).

E-mail addresses: zamaniali@gmail.com (A. Zamani), zhicanqu@gmail.com (Z. Qu).

protein kinase (ERK); and p38 mitogen-activated protein kinase (p38 MAPK). Four primary endothelial cell lines were used: human umbilical vein endothelial cells (HUVECs), human microvascular endothelial cells (HMVECs), human pulmonary artery endothelial cells (HPAECs), and human aortic endothelial cells (HAECs). These cells were examined for kinase activation upon 5-HT stimulation with western blot analyses.

5-HT (1 μ M) could activate all seven signaling kinases (Src, PI3K, AKT, mTOR, p70S6K, ERK, and p38 MAPK; Fig. 1A). In healthy human subjects, the mean level of 5-HT was approximately 10 pM in plasma, 1 μ M in serum, and 400 μ M in platelets [19]. Serum contains factors released from blood cells after the coagulation process, and plasma does not. Activation of these kinases was further examined for 5-HT dose responses in the range 1 nM–10 μ M. 5-HT stimulated dose-dependent activation of the seven signaling kinases (Fig. 1B). This result demonstrated that 5-HT could activate a set of phosphorylation signaling kinases in human endothelial cells under physiologically relevant concentrations.

2.2. 5-HT receptor 1B mediates activation of signaling kinases

Studies have shown that the diverse functions of 5-HT are dependent upon interactions with its specific receptor subtypes selectively expressed in different cell types [20,21]. Five 5-HT receptor subtypes (HTR1A, HTR1B, HTR1D, HTR2B, HTR7) have been identified for their expression in human endothelial cells [9]. Taking advantage of agents and probes developed for selectively targeting each serotonin receptor, specific antagonists against the five 5-HT receptors were applied to assess their role

in mediating 5-HT signaling. The highly selective HTR1B antagonist SB216641 [22] blocked the 5-HT-provoked activation of Src, PI3K, AKT, mTOR, p70S6K, and ERK, whereas the activation of p38 MAPK was not affected by treatment with SB216641 (Fig. 2A). In contrast to the HTR1B antagonist that diminished activation of six signaling kinases, the antagonists against the HTR2B and HTR7 reduced p38 kinase activation but did not affect activation of any other kinases tested. In addition, the HTR1A antagonist abrogated activation of p70S6K but did not affect any of the others (Fig. 2A).

The results detailed above suggested that HTR1B might be the main receptor subtype that mediates 5-HT-activated signaling kinases. Hence, a highly selective HTR1B agonist, CP93129 dihydrochloride [23], was applied for further examination. CP93129 could closely resemble the effects of 5-HT on the activation of kinases in human endothelial cells (Fig. 2B). In addition, the HTR1B antagonist SB216641 could block the 5-HT- and CP93129-stimulated kinase activation of Src, PI3K, AKT, mTOR, p70S6K, and ERK. Consistently, SB216641 had no effect against the activation of p38 MAPK stimulated by 5-HT or CP93129. These results provide further evidence in support of the notion that HTR1B is the main membrane receptor that mediates the kinase activation induced by 5-HT.

2.3. GPCRi transduces 5-HT signaling to activate signaling kinases

The 5-HT receptor HTR1B has been characterized as an inhibitory subtype of a G-protein-coupled receptor (GPCRi) [24,25]. Activation of GPCR leads to its interaction with a G-protein complex which comprises three subunits (α , β , γ) and results in dissociation

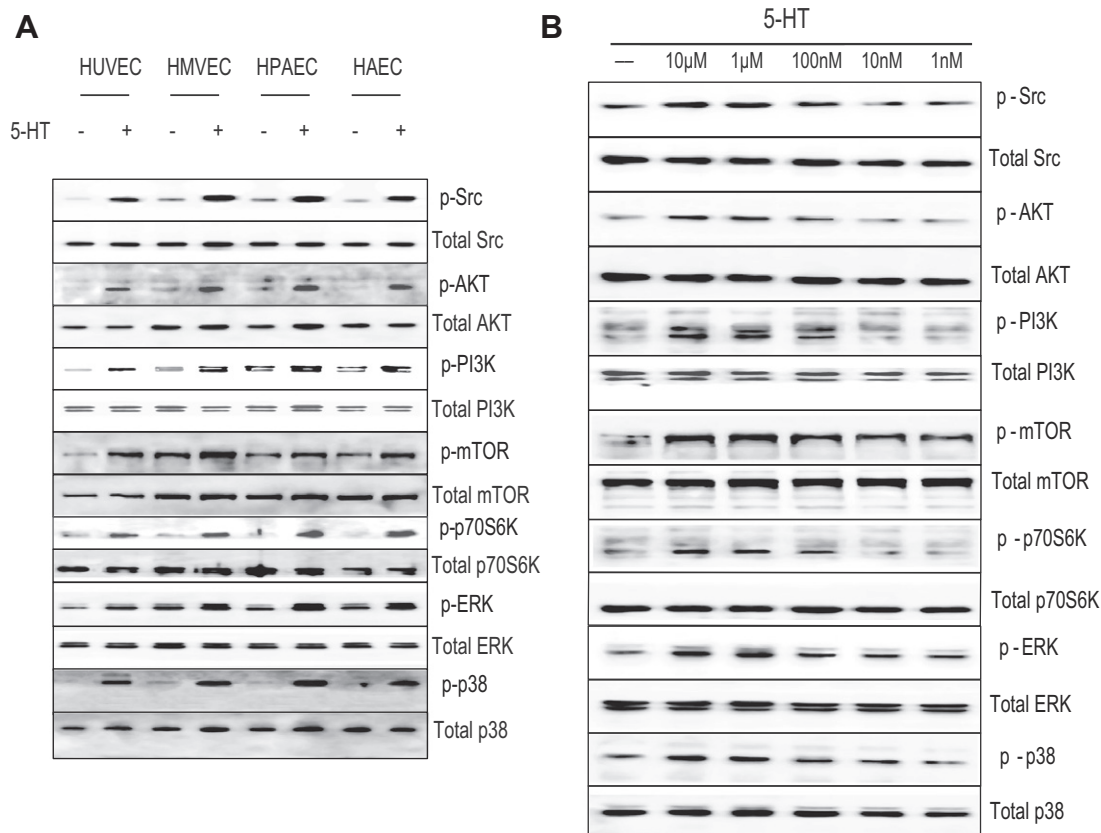


Fig. 1. 5-HT activates signaling kinases in human endothelial cells (A) Human endothelial cell lysates were prepared after 5-HT stimulation (1 μ M) for 15 min. Western blotting was conducted with specific antibodies as indicated to reveal the activation status of signaling kinases. (HUVEC, human umbilical vein endothelial cell; HMVEC, human microvascular endothelial cell; HPAEC, human pulmonary artery endothelial cell; HAEC, human aortic endothelial cell). (B) Western blotting of endothelial cell lysates for 5-HT dose-response activation of kinases. HUVECs were stimulated with 5-HT at different concentrations as indicated for 15 min before preparation of the cell lysates.

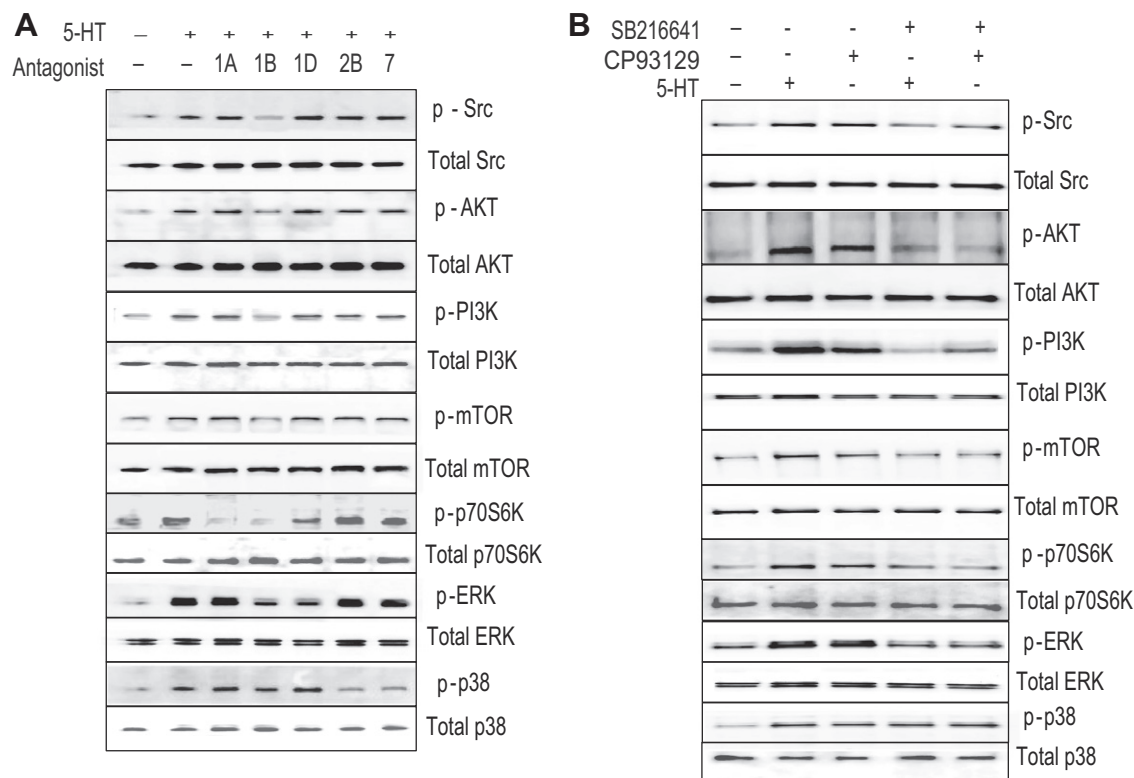


Fig. 2. 5-HT receptor 1B mediates the activation of signaling kinases (A) HUVEC cultures were treated with selective antagonists at 100 nM as indicated for 1 h followed by 5-HT stimulation at 100 nM for 15 min. (1A is the HTR1A antagonist WAY100635, 1B is the HTR1B antagonist SB216641, 1D is the HTR1D antagonist BRL15572, 2B is the HTR2B antagonist SB200646, and 7 is the HTR7 antagonist SB269970). Western blotting of the cell lysates was conducted with the specific antibodies as indicated. (B) Western blotting of HUVEC lysates, which were prepared after treatment with 5-HT or a HTR1B agonist (CP93129) at 100 nM for 15 min. In the case of the antagonist, HUVEC cultures were pre-treated with the HTR1B antagonist SB216641 at 100 nM for 1 h before stimulation with 5-HT or a HTR1B agonist (100 nM) for 15 min.

of the α subunit from the $\beta\gamma$ subunit complex. For GPCR-mediated transmembrane signal transduction, the activated α_i subunit inhibits adenylate cyclase (AC) to reduce production of cyclic adenosine monophosphate (cAMP), and the activated $\beta\gamma$ subunit catalyzes the cleavage of phosphatidylinositol bisphosphate (PIP2) to generate inositol triphosphate (IP3) [26]. cAMP and IP3 are considered to be important secondary messengers in GPCR signal transduction. Agonists of 5-HT and HTR1B have shown similar dose-dependent cAMP down-regulation and IP3 up-regulation in endothelial cells [9]. The protein-based exotoxin pertussis toxin (PTX) can specifically “lock” the α_i subunit to its inactive state [27]; and the xanthine compound gallein selectively binds to the $\beta\gamma$ subunit complex with high affinity to block $\beta\gamma$ -dependent signaling [28]. Using PTX and gallein as biological probes, α_i - or $\beta\gamma$ -dependent kinase activations were examined. By blocking α_i or $\beta\gamma$, the 5-HT-evoked activations of AKT, mTOR, and p70S6K were diminished (Fig. 3A). Activation of p38 MAPK was affected by blocking $\beta\gamma$ but not by blocking α_i . This observation was consistent with the result that activation of p38 MAPK was not through GPCR HTR1B (Fig. 2A). Activation of PI3K was blocked only by a α_i blocker.

2.4. 5-HT stimulates a phosphorylation signaling cascade in endothelial cells

Several studies have shown that the non-receptor tyrosine kinase Src has important roles in numerous cellular processes, such as proliferation, survival, and cytoskeleton rearrangement [29]. Stimulation by 5-HT activated Src in endothelial cells through its receptor subtype HTR1B (Figs. 1 and 2). The α_i blocker PTX could block Src activation, whereas the $\beta\gamma$ blocker gallein could not, suggesting that Src activation was mediated by α_i activation as an up-

stream event (Fig. 3A). The Src-selective inhibitor PP2 was then used to ascertain if other kinase activations were dependent upon Src activation. Blocking Src activation with PP2 diminished the activation of PI3K, AKT, mTOR, p70S6K, and ERK (Fig. 3A). This finding suggested that activations of these kinases were downstream of Src activation in 5-HT phosphorylation signaling. Activation of p38 MAPK was not affected by the blockade of Src activation, suggesting that p38 MAPK was not in the Src downstream signaling pathway.

To elucidate the sequential signal transduction events of 5-HT signaling in endothelial cells, the basal effects of selective kinase inhibitors in endothelial cultures were characterized. Then, optimized concentrations of each inhibitor were obtained for examination of 5-HT-induced signal transduction. The PI3K-selective inhibitor LY294002 blocked activation of PI3K, AKT and mTOR (Fig. 3B). Therefore, PI3K activation was an upstream event of AKT and mTOR activation. The mTOR inhibitor rapamycin diminished only the activation of mTOR, and the MEK inhibitor U0126 blocked only ERK activation. Interestingly, neither inhibitors of PI3K, mTOR, and MEK alone could block 5-HT-activated p70S6K. This finding suggested that p70S6K possibly receives multiple inputs from different signaling routes evoked by the 5-HT stimulus in human endothelial cells.

Based on the results described above, a working model of 5-HT signaling in endothelia is illustrated in Fig. 4. Mediated by GPCR membrane receptors, 5-HT stimulates more than one intracellular phosphorylation signaling pathway in human endothelial cells. One of the pathways is Src/PI3K/AKT/mTOR, which is mediated by HTR1B. In addition, 5-HT also activated p38 MAPK, ERK, and p70S6K. These results suggest that the activation of ERK and p70S6K was mediated by HTR1B, whereas activation of p38 MAPK

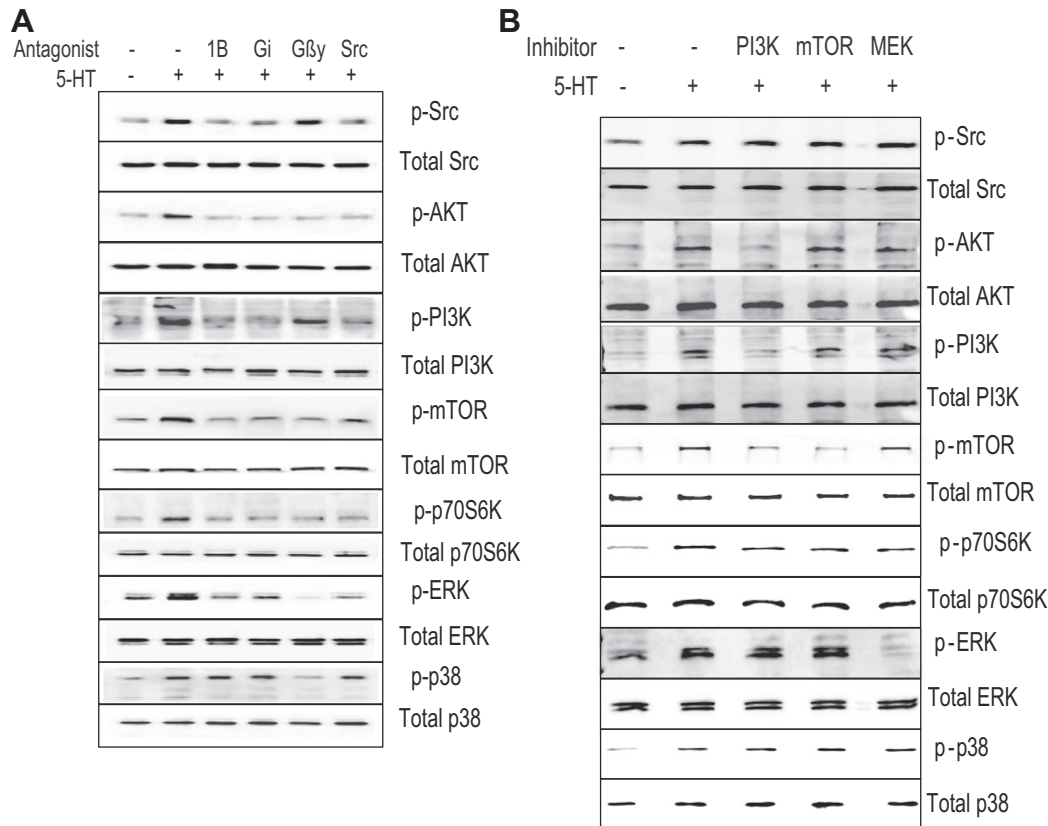


Fig. 3. 5-HT stimulates phosphorylation signaling in endothelial cells (A) HUVEC cultures were treated with the HTR1B antagonist SB216641 at 100 nM (1B), PTX at 1 μ g/mL (Gi), gallein at 10 μ M (G β γ), and PP2 at 1 μ M (Src) for 1 h followed by 5-HT stimulation at 100 nM for 15 min. Western blotting of the cell lysate was conducted with the specific antibodies as indicated. (B) HUVEC cultures were treated with the PI3 K inhibitor LY294002 at 10 μ M (PI3 K), mTOR inhibitor (rapamycin) at 10 nM (mTOR), and MEK inhibitor (U0126) at 100 nM (MEK) for 1 h and followed by 5-HT stimulation at 100 nM for 15 min. Western blotting of the cell lysates was conducted with the specific antibodies as indicated.

may not be through HTR1B. Serine/threonine p70S6K has been reported to be activated by numerous signaling intermediates (including PI3K and mTOR), and the role of p70S6K and its regulation in 5-HT signaling needs to be investigated further.

2.5. 5-HT and VEGF stimulate the same set of signaling kinases

VEGF is considered to be an important angiogenic factor. VEGF has been reported to activate several kinases for signal transduction in endothelia, including the seven kinases examined in the present study. In the tumor environment, VEGF and 5-HT can interact with adjacent endothelial cells and activate tumor angiogenesis. We examined kinase activation in endothelial cells stimulated by VEGF and 5-HT simultaneously under identical experimental conditions. In healthy human subjects, the mean levels of VEGF were 42 pg/mL in plasma and 173 pg/mL in serum [30]. However, VEGF above its human serum concentration (200 pg/mL) could not stimulate kinase activation in endothelial cells (data not shown). VEGF at a concentration range of 10–25 ng/mL has been used for the activation of kinases [31]. VEGF and 5-HT could activate the endothelial signaling kinases Src, PI3K, AKT, mTOR, p70S6K, ERK, and p38 MAPK, whereas only VEGF could activate the VEGF receptor VEGFR2 (Fig. 5). The VEGFR2 tyrosine kinase inhibitor sunitinib could block (or significantly reduce) VEGF-provoked kinase activations, whereas it did not have a significant effect on the kinase activation stimulated by 5-HT. Conversely, the 5-HT receptor antagonist SB216641 blocked the 5-HT-provoked kinase activation of Src, PI3K, AKT, mTOR, p70S6K, and ERK, and had no effect on VEGF-stimulated activation of these signaling kinases.

Importantly, the 5-HT concentration in this experiment was 100 nM, which was only one-tenth of the 5-HT concentration in human serum, and the VEGF concentration was 20 ng/mL, which was not only much higher than that observed in human serum, but also much higher than the mean VEGF level of 0.3 ng/mg protein measured in resected tumor samples from 2800 cancer patients [30]. This result suggested that, when considering physiologically relevant conditions, 5-HT might be a more potent and relevant angiogenic factor in supporting tumor progression. In addition, although the action is through different membrane receptors, VEGF and 5-HT activate an identical set of signaling kinases, demonstrating that the downstream angiogenic signaling pathways of VEGF and 5-HT are crossed at several points (or partially overlapped). Therefore, if blocking one angiogenic factor at its upstream signal (e.g., blocking VEGF or VEGFR2), the effect might be compensated at the downstream signal by the signaling network stimulated by other angiogenic factors.

3. Discussion

In the recent years, clinical trials focusing on VEGF neutralization antibody or inhibitors of the VEGF receptor (VEGFR2) have shown limited and transient efficacy in cancer treatment [11,14]. This has challenged the current belief of VEGF being a major tumor angiogenic factor [13,32]. Platelet aggregation and blood coagulation are common features in tumor environments. Platelet activation leads to a significant release of 5-HT in tumor environment. The released 5-HT can directly interact with adjacent endothelial cells and lead to activation of angiogenic pathways. Although an

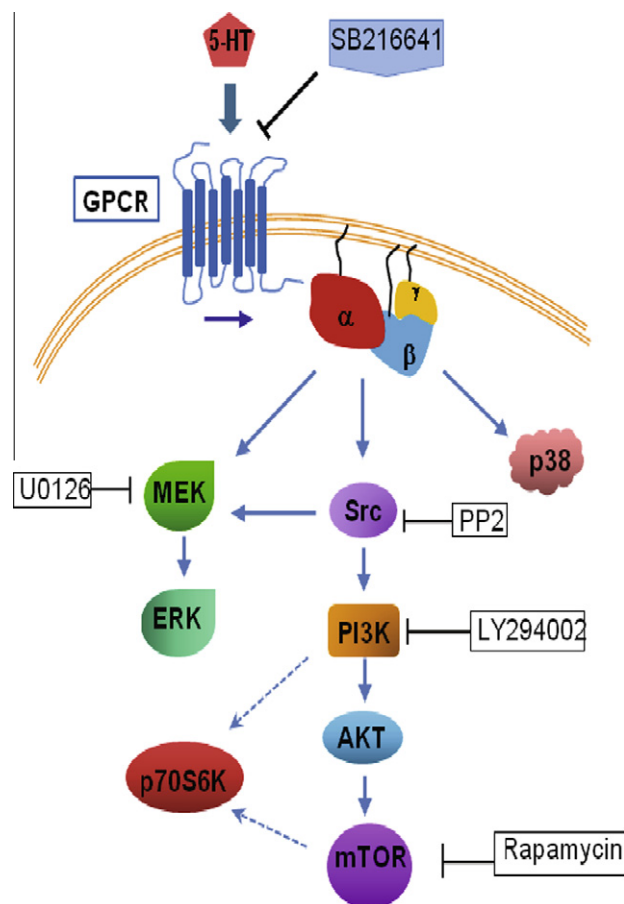


Fig. 4. Working model of 5-HT signal transduction in endothelial cells.

association between platelet activation and cancers has been known for over hundred years [5,33], however, due to its lack of a nucleus, the impact of platelets and its stored 5-HT are overlooked by high-impact, meta-analysis studies focusing on the genomic profiling of tumors. In the present study, we examined the stimulatory effects of 5-HT on activation of a set of signaling kinases in human endothelia, and compared them with those seen with VEGF stimulation. 5-HT at concentrations lower than those seen in human serum could activate angiogenic signaling kinases, whereas VEGF at its concentration in human serum (or even at its concentration in tumors) could not activate the same signaling kinases. These findings suggested that, when considering physiologically relevant conditions, 5-HT might be a more potent angiogenic factor than VEGF in driving tumor angiogenesis. 5-HT-stimulated angiogenesis may also serve as one of mechanisms underlying the resistance of current VEGF-targeting anti-angiogenic therapy in cancer.

4. Materials and methods

4.1. Cell cultures, inhibitors and chemical agents

HUVECs, HPAECs, HMVECs, and HAECs (Lonza, Walkersville, MD, USA) were maintained in culture with a full growth medium as Endothelial Basal Medium (EBM) (Lonza) supplemented with 2% fetal bovine serum (FBS), 0.4% bovine brain extract (Lonza) and 1/1000 GA-1000 (gentamicin, amphotericin-B). Serotonin creatinine sulfate monohydrate (5-HT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). CP93129 dihydrochloride, WAY100635

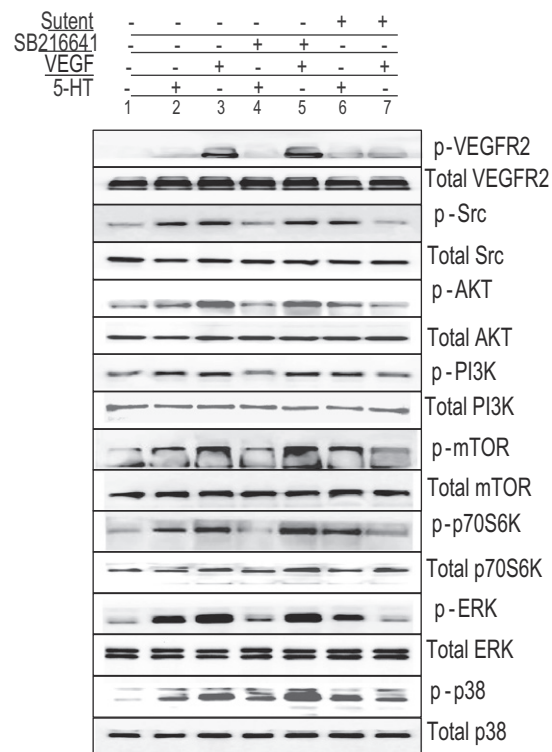


Fig. 5. 5-HT and VEGF stimulate the same set of signaling kinases. HUVEC lysates were prepared after treatment with 5-HT at 100 nM or VEGF at 10 ng/mL for 15 min. In the case of antagonists, HUVEC cultures were pre-treated with the HTR1B antagonist (SB216641) at 100 nM or sunitinib at 100 nM for 1 h and followed by stimulation with 100 nM 5-HT or 10 ng/mL VEGF for 15 min. Western blotting of the cell lysates was conducted with the specific antibodies as indicated.

maleate, SB216641 hydrochloride, BRL15572 hydrochloride, SB206553 hydrochloride, SB269970 hydrochloride, PTX, gallein, PP2, LY294002, U0126 and rapamycin were obtained from Tocris Biosciences (Ellisville, MO, USA). SU11248 (sunitinib) was obtained from Pfizer (New York, NY, USA). Recombinant human VEGF (165 amino acids) was from R&D Systems (Minneapolis, MN, USA).

4.2. Endothelial stimulation experiments and western blotting analyses

For all endothelial stimulation experiments, cell cultures were first switched to Basic Medium (EBM plus 0.1% FBS) for 1-h starvation, and different treatments were then undertaken. Only primary endothelial cultures between passages 3 and 5 (confluence, ~90%) were used for 5-HT stimulation experiments. Endothelial cell lysates were prepared with cell lysis buffer (10 mM Tris-HCl buffer at pH 8, 100 mM sodium phosphate, 3 M urea, 1% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol). Cell lysates under different treatment conditions were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blots were conducted with specific primary and secondary antibodies, and visualized with high-sensitivity enhanced chemiluminescence (ECL) (Amersham-Pharmacia, Uppsala, Sweden). All primary antibodies were obtained from Cell Signaling (Danvers, MA, USA) as specific antibodies against the kinase proteins of VEGFR2, Src, PI3K, AKT, ERK, mTOR, p70S6K, and p38 MAPK; and antibodies against the phospho-specific sites of these kinases as anti-phospho-VEGFR2 (Tyr 951), phospho-Src (Tyr 416), phospho-PI3K (Tyr 458/199), phospho-AKT (Thr 308), phospho-ERK (Thr 202/Tyr 204), phospho-mTOR (Ser 2448), phospho-p70S6K (Thr 421/Ser 424), and phospho-p38 (Thr180/Tyr182). Each of

the western blotting experiments were conducted at least two times to confirm the study results.

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